High-sugar, but not high-fat, food activates supraoptic oxytocin neurones in the male rat

High-Sugar, but Not High-Fat, Food Activates Supraoptic Nucleus Neurons in the Male Rat

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Oxytocin is a potent anorexigen and is believed to have a role in satiety signaling. We developed rat models to study the activity of oxytocin neurons in response to voluntary consumption or oral gavage of foods using c-Fos immunohistochemistry and *in vivo* electrophysiology. Using c-Fos expression as an indirect marker of neural activation, we showed that the percentage of magnocellular oxytocin neurons expressing c-Fos increased with voluntary consumption of sweetened condensed milk (SCM). To model the effect of food in the stomach, we gavaged anesthetized rats with SCM. The percentage of supraoptic nucleus and paraventricular nucleus magnocellular oxytocin-immunoreactive neurons expressing c-Fos increased with SCM gavage but not with gastric distention. To further examine the activity of the supraoptic nucleus, we made *in vivo* electrophysiological recordings from SON neurons, where anesthetized rats were gavaged with SCM or single cream. Pharmacologically identified oxytocin neurons responded to SCM gavage with a linear, proportional, and sustained increase in firing rate, but cream gavage resulted in a transient reduction in firing rate. Blood glucose increased after SCM gavage but not cream gavage. Plasma osmolarity and plasma sodium were unchanged throughout. We show that in response to high-sugar, but not high-fat, food in the stomach, there is an increase in the activity of oxytocin neurons. This does not appear to be a consequence of stomach distention or changes in osmotic pressure. Our data suggest that the presence of specific foods with different macronutrient profiles in the stomach differentially regulates the activity of oxytocin neurons. **(*Endocrinology* 158: 2200–2211, 2017)**

Oxytocin is an anorexigen synthesized in neurons of the hypothalamic supraoptic nucleus (SON) and paraventricular nucleus (PVN). The SON and PVN contain magnocellular oxytocin neurons that project to the posterior pituitary and secrete their products into the circulation. These neurons can also secrete large amounts of oxytocin from their dendrites to act at multiple and diverse brain targets (1–3); a few also send axonal projections to forebrain areas, including the amygdala (4). In addition to magnocellular oxytocin cells, the PVN contains a population of parvocellular oxytocin neurons that project centrally. Many of these neurons project to caudal brainstem regions, including the nucleus of the solitary tract (NTS), a region involved in regulation of the gastrointestinal vago-vagal reflex and gastric emptying (5, 6). Oxytocin fibers also innervate the arcuate nucleus (ARC) (7).

There is robust evidence from rodent models that oxytocin is powerfully anorexigenic. Central oxytocin administration dose-dependently decreases voluntary food intake, and this is dependent on the oxytocin receptor (8–10). There is also considerable evidence of a role for both magnocellular and parvocellular oxytocin neurons in the physiological response to food consumption. Expression of the immediate early gene c-Fos in magnocellular oxytocin neurons of the SON is increased by food intake and associated stimuli, such as refeeding after fasting (11), palatable food consumption (12), changes in osmotic pressure (13), gastric distention (14), and systemic administration of the satiety mediators

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Abbreviations: ARC, arcuate nucleus; CCK, cholecystokinin; GLP-1, glucagonlike peptide 1; IgG, immunoglobulin G; IP, intraperitoneally; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus; SCM, sweetened condensed milk; SON, supraoptic nucleus; TH, tyrosine hydroxylase; VMH, ventromedial nucleus of the hypothalamus; α-MSH, α-melanocyte stimulating hormone.

cholecystokinin (CCK) (15), secretin (16), and leptin (17). Magnocellular oxytocin neurons coexpress several other anorexigenic peptides, most conspicuously CCK (18) and nesfatin (19). They express melanocortin MC4 receptors and respond to direct application of α-melanocyte stimulating hormone (α-MSH) by releasing oxytocin from their dendrites (20). Oxytocin receptors are expressed at a very high density in the ventromedial nucleus of the hypothalamus (VMH) and in the amygdala, which are brain regions that contain very few oxytocin fibers and hence are thought to be likely targets of dendritically released oxytocin, acting as a neurohormone. More recently, it has been suggested that oxytocin signaling may be especially relevant to hedonic food choice and consumption, particularly sugar consumption (21–23).

The effect of food or nutrient consumption on the electrical activity of oxytocin neurons is relatively unexplored, either via gut-derived hormonal or vagal signaling or via oro-sensory signaling (12, 24). Therefore, we hypothesized that the activity of oxytocin neurons could be increased *in vivo* by gut-brain signaling when food [sweetened condensed milk (SCM)] is consumed voluntarily or delivered directly into the stomach by gavage in anesthetized rats. We tested this using c-Fos immunohistochemistry and *in vivo* electrophysiology, recording from neurons in the SON. Furthermore, we investigated whether the delivery of an isocaloric food with a different macronutrient profile to SCM (low-sugar, high-fat single cream) directly into the stomach differentially influences the electrical activity of oxytocin neurons.

Materials and Methods

All behavioral and surgical procedures were carried out under UK Home Office regulations and after approval from the local ethical committee. Male adult Sprague-Dawley rats (8 to 10 weeks old) were kept on a 12-h light cycle (lights on at 7:00 AM) at 20 ± 1°C and given *ad libitum* access to water and food (RM1; Special Diet Services, Essex, UK). A volume of 5 mL of SCM (diluted 50% v/v in water; 40.8 kJ, 0.24 g fat, 1.68 g sugar) (Nestlé, UK) or single cream (40.6 KJ, 0.955 g fat, 0.11 g sugar) was used. Single cream was used because it is isocaloric to SCM but has a higher fat and lower sugar content. All reagents were obtained from Sigma (UK) unless otherwise stated.

Experiment 1: c-Fos–like immunohistochemistry after voluntary SCM consumption

Sixteen male rats were singly housed. Eight rats were assigned to a control group and were not given SCM. Eight rats were schedule-fed SCM, receiving 15 minutes of SCM access at the same point in the early light phase for 8 days ("SCM access" group). On day 8, six rats consumed the SCM. Two rats did not consume the SCM and were excluded from further analysis.

On the final day of SCM scheduled feeding, 1 h after SCM access, rats were euthanized with an overdose of pentobarbital given intraperitoneally (IP) followed by transcardial perfusion with ice-cold saline containing 0.012% w/v heparin followed by 4% w/v paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, postfixed, cryo-protected, frozen on dry ice, and cut serially on a freezing microtome at 44 μm in the coronal plane. All sections were processed for c-Fos–like immunoreactivity using an anti–c-Fos rabbit primary antibody (1:100,000) (226 003; Synaptic Systems, Germany) and biotinylated horse anti-rabbit immunoglobulin G (IgG) secondary antibody (1:500) (BA-1100; Vector Laboratories, UK) as described previously (25). A subset of sections were double stained using anti–oxytocin-neurophysin (1:5000) (PS 38; supplied by H. Gainer, Bethesda, MD) mouse primary antibody or anti-tyrosine hydroxylase (TH) (1:20,000) (MAB318; Merck Millipore, UK) mouse primary antibody and biotinylated horse anti-mouse IgG secondary antibody (1:500) (BA-2000; Vector Laboratories). Table 1 lists the antibodies used. Positive control brain sections from rats given a hyperosmotic stimulus (3.5 M NaCl 600 μL/kg, IP) were used. We observed dense c-Fos expression in the SON and PVN, indicating that the primary antibody was selective and sensitive. Negative control rat brain sections incubated with preimmunized rabbit (for c-Fos) or mouse (for oxytocin-neurophysin or TH) serum showed no immunoreactivity. Preabsorption control rat brain sections incubated with preabsorbed c-Fos primary antibody (c-Fos antibody incubated overnight with an excess of purified c-Fos peptide) (226-0P; Synaptic Systems) also showed no immunoreactivity.

Brain sections were mounted on gelatin-coated slides and dehydrated in alcohol followed by xylene. Coverslips were applied using DPX mounting medium and viewed using a reflected light microscope (DMR; Leica, UK). Regions of interest were identified with reference to a brain atlas (26). Nuclei positive for c-Fos were counted bilaterally, and the mean number of c-Fos–positive nuclei per section was calculated for each rat before a group mean was calculated. For quantification of the percentage of oxytocin or TH-immunoreactive cells expressing c-Fos, the nucleus of each labeled cell was assessed for c-Fos immunoreactivity. Magnocellular and parvocellular regions of the PVN were distinguished as described previously (15). Because the SON only contains oxytocin and vasopressin neurons, the number of c-Fos–positive nuclei in SON non–oxytocin-immunoreactive cells was also determined to quantify c-Fos expression in putative vasopressin cells. Counting was carried out under blinded conditions by an experienced experimenter. The same regions of the SON, PVN, and NTS were counted for each brain. For c-Fos expression in the SON, sections between bregma −0.72 and −1.08 mm (26) were counted (four sections per brain). For c-Fos expression in oxytocin cells of the SON, sections at bregma points −0.84 and −0.96 mm were counted (two sections per brain). For c-Fos expression in the PVN, all sections between bregma −1.56 and −1.92 mm were counted (three sections per brain). For c-Fos expression in oxytocin cells of the PVN, sections at bregma points −1.72 and −1.80 mm were counted (two sections per brain). For c-Fos expression in the NTS, all sections between bregma −13.68 and −14.16 mm were counted (four sections per brain). For c-Fos expression in TH cells of the NTS, sections at bregma points −13.92 and −14.04 mm were counted (two sections per brain). Differences in c-Fos expression and

Table 1. Antibodies Used

Peptide/Protein Target	Antigen Sequence	Name of Antibody	Manufacturer, Catalog No., or Name of Source	Species Raised in; Monoclonal or Polyclonal	Dilution Used	Research Resource Identifier
c-Fos	Unknown	Anti–c-Fos	Synaptic Systems, 226 003	Rabbit; polyclonal	1 in 100,000	AB_1966442
Oxytocin-neurophysin	Unknown	PS 38	Supplied by H. Gainer	Mouse; monoclonal	1 in 5000	AB_2315026
Tyrosine hydroxylase	Unknown	Anti-tyrosine hydroxylase antibody, clone LNC1	Merck Millipore, MAB318	Mouse; monoclonal	1 in 20,000	AB_2201528
Rabbit IgG	Unknown	Biotinylated horse anti-rabbit IgG Antibody	Vector Laboratories, BA-1100	Horse	1 in 500	AB_2336201
Mouse IgG	Unknown	Biotinylated horse anti-mouse IgG antibody	Vector Laboratories, BA-2000	Horse	1 in 500	AB_2313571

percentage of oxytocin or TH-immunoreactive neurons expressing c-Fos–positive nuclei between groups were determined using Mann-Whitney tests.

Experiment 2: c-Fos–like immunohistochemistry after SCM gavage in anesthetized rats

Rats were fasted overnight for 16 to 18 hours to empty the stomach. Anesthesia was induced using isoflurane followed by an intraperitoneal injection of pentobarbital sodium (60 mg/kg). Once rats were deeply anesthetized, a femoral vein was cannulated to allow the maintenance of anesthesia by continuous infusion of sodium pentobarbital (18.6 mg/kg/h) in 0.9% w/v saline (10 mL/kg/h). Rats were tracheotomized, and a plastic gavage tube (FTP-13-150; Solomon Scientific) was inserted via the mouth into the stomach. After at least 2 hours, 5 mL of SCM was infused slowly (0.14 mL/min) into the stomach via gavage tube (n = 8; "SCM gavage"). A second group of rats were subjected to a sham gavage. These animals were treated the same as those receiving SCM; however, nothing was infused (n = 8; "sham gavage"). To control for gastric distention, a final group of rats were subjected to the inflation of a fine latex balloon attached to the bottom of the gavage tube (n = 9; "balloon gavage"). The balloon was inflated with 5 mL water at the same rate as the SCM gavage.

One hour after the end of sham, balloon, or SCM gavage, rats were given an overdose of pentobarbital sodium via the femoral vein cannula, perfused transcardially, and processed for immunochemistry as described previously. Differences in c-Fos expression and percentage of oxytocin or TH-immunoreactive neurons expressing c-Fos–positive nuclei between groups were determined using one-way analysis of variance with Bonferroni.

Experiment 3: Blood sampling during SCM or cream gavage in anesthetized rats

In a separate cohort of male adult pentobarbital-anesthetized rats undergoing SCM gavage, blood samples were collected via a jugular cannula filled with heparinized saline (50 U/mL) into EDTA-coated tubes at time −20, 0, 20, 60 minutes with respect to start of sham (n = 3) or SCM (n = 4) gavage. An additional group of rats were gavaged with 5 mL of single cream (n = 5), and blood was sampled. At each time point, blood glucose was measured using a glucose meter (ACCU-CHEK Aviva; Roche, Germany). Samples were centrifuged at 13,000 RPM at room temperature for 10 minutes, and plasma was collected. Plasma insulin was measured by ELISA (rat/mouse insulin ELISA) (EZRMI-13K; Merck Millipore). Intra-assay variability was <9%. Plasma osmolarity was measured using an osmometer (Model 3300; Advanced Instruments, Inc.,). Plasma sodium was measured using a flame photometer (PFP7; Jenway, UK). Plasma samples were diluted in deionized water (1:500), and the sodium concentrations were interpolated from a standard curve (Na, 0 to 10 mg/L). Friedman tests with Dunn multiple comparisons were used to compare differences in blood glucose, plasma insulin, plasma osmolarity, and plasma sodium over time within the sham, cream, and SCM gavage groups.

Experiment 4: *In vivo* electrophysiology during SCM or cream gavage in anesthetized rats

c-Fos–like immunoreactivity is only an indirect indicator of neural activity, and its expression is not necessarily associated with an increase in action potential firing (20). To examine neuronal activity directly, we used *in vivo* electrophysiology to record from oxytocin and vasopressin neurons in the SON of anesthetized rats during SCM gavage. SON oxytocin cells were also recorded from during single cream gavage.

Adult male rats were anesthetized with urethane (1.25 g/kg, IP), and a femoral vein and the trachea were cannulated. The SON and neural stalk of the pituitary were exposed by transpharyngeal surgery. A gavage tube was inserted orally into the stomach to allow infusion of 5 mL SCM or cream directly into the stomach using a syringe pump (0.14 mL/min). A bipolar stainless steel electrode was placed on the neural stalk, and a glass microelectrode filled with 0.9% NaCl was introduced into the SON under direct visual control. Single SON neurons were identified antidromically and recorded using conventional extracellular recording techniques. Oxytocin cells were distinguished from vasopressin cells by their opposite response to intravenous CCK (20 μg/kg; octapeptide, sulfated) (Tocris, UK) (27, 28).

We set a change of 0.5 spikes/s as a threshold above which the cell was considered as responsive to the substance applied. Cells in which the change in mean firing rate was less than ±0.5 spikes/s after gavage were categorized as nonresponding cells. For cells that showed a change of >0.5 spikes/s after gavage, the mean activity in 30-second bins in the 10 minutes before gavage was compared with activity in the 50-minute period between 10 and 60 minutes after the start of gavage, using Student t test, accepting $P < 0.001$ as significant. The mean change in firing rate in the period 10 to 60 minutes after the start of gavage was tested for the whole population of oxytocin cells to check whether, as a population response, this change was significantly different from 0. For this, we used a Wilcoxon signed rank test, accepting $P < 0.05$ as significant.

Results

Experiment 1: c-Fos–like immunohistochemistry after voluntary SCM consumption

We quantified c-Fos expression as a marker of neural activation in response to the voluntary consumption of SCM. In the SON, the percentage of oxytocin neurons expressing c-Fos was significantly greater in rats voluntarily consuming SCM in comparison with controls (control, 3 ± 1%; SCM, 23 ± 3%; $P = 0.0012$) [Fig. 1(a) and 1(b)]. The SON contains only oxytocin and vasopressin neurons, so we classified cells that were not oxytocin immunoreactive as putative vasopressin neurons. In comparison with controls, there was no significant difference in the number of putative vasopressin cells expressing c-Fos when rats voluntarily ate SCM (control, 16 ± 2 nuclei/section; SCM, 22 ± 1 nuclei/section; $P = 0.14$).

In the PVN, the percentage of magnocellular oxytocin neurons expressing c-Fos significantly increased with voluntary SCM consumption in comparison with controls (control, 9 ± 1%; SCM, 28 ± 2%; $P = 0.0012$) [Fig. 1(c) and 1(e)]. There was no significant difference in c-Fos expression in parvocellular PVN oxytocin neurons between the control and SCM consumption groups (control, 5 ± 1%; SCM, 8 ± 2%; $P = 0.18$) [Fig. 1(d)].

We also studied other appetite-related brain nuclei. In the NTS, an important site in the relay of peripheral signaling to the hypothalamus, the percentage of TH containing neurons expressing c-Fos significantly increased after the voluntary consumption of SCM (control, 4 ± 0.4%; SCM, 13 ± 4%; $P = 0.02$).

In the ARC and VMH, there were no significant differences in c-Fos expression after voluntary SCM consumption in comparison with controls (ARC: control, 78 ± 5 nuclei/section; SCM, 118 ± 23 nuclei/section; $P = 0.6$; VMH: control, 13 ± 3 nuclei/section; SCM, 15 ± 2 nuclei/section; $P = 0.84$).

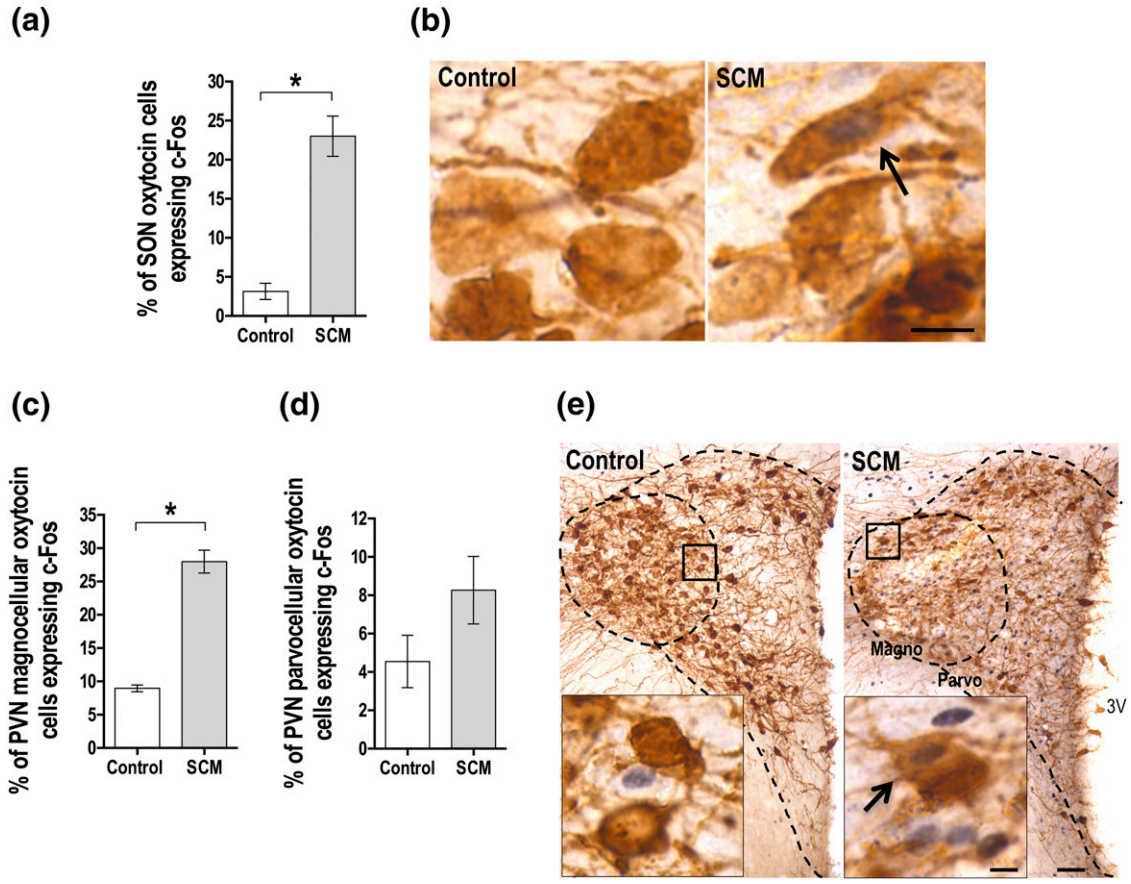

Figure 1. c-Fos–like immunohistochemistry in oxytocin neurons of the SON and PVN in response to the voluntary consumption of SCM. Data shown as means ± standard error of the mean. *$P < 0.05$. (a) Percentage of SON oxytocin-immunoreactive cells expressing c-Fos–positive nuclei per brain section for control and SCM consumption groups ($P = 0.0012$, Mann-Whitney test). (b) Representative images of c-Fos expression within SON oxytocin-immunoreactive cells for control and SCM consumption groups. Bregma −0.84 mm (26). Scale bar represents 10 μm. (c) Percentage of PVN magnocellular oxytocin-immunoreactive cells expressing c-Fos positive nuclei per brain section for control and SCM consumption groups ($P = 0.0012$, Mann-Whitney test). (d) Percentage of PVN parvocellular oxytocin-immunoreactive cells expressing c-Fos–positive nuclei per brain section for control and SCM consumption groups (Mann-Whitney test, $P = 0.17$). (e) Representative images of c-Fos expression within PVN oxytocin-immunoreactive cells for control and SCM consumption groups. Bregma −1.72 mm (26). Main scale bar represents 50 μm; inset scale bar represents 10 μm. 3V, 3rd ventricle; Magno, magnocellular PVN; Parvo, parvocellular PVN.

Experiment 2: c-Fos–like immunohistochemistry after SCM gavage in anesthetized rats

To further investigate this c-Fos response, we developed an *in vivo* model to quantify c-Fos expression as a marker of neural activation in response to sham, balloon, and SCM gavage in oxytocin neurons of the SON and PVN.

In the SON, the percentage of oxytocin neurons that expressed c-Fos was significantly higher with SCM gavage than in either the sham control group or the balloon gavage group (sham gavage, 19 ± 1%; balloon gavage, 22 ± 3%; SCM gavage, 38 ± 7%; $P = 0.02$ and $P = 0.04$, respectively) [Fig. 2(a) and 2(b)], and the balloon gavage group was not significantly different from the sham control group ($P > 0.99$) [Fig. 2(a)]. There was no significant difference in SON c-Fos expression in putative vasopressin cells between sham gavage and balloon gavage groups (sham gavage, 87 ± 9 nuclei/section; balloon gavage, 112 ± 18 nuclei/section; $P = 0.8$) or between sham gavage and SCM gavage groups (sham gavage, 87 ± 9 nuclei/section; SCM gavage, 142 ± 18 nuclei/section; $P = 0.08$).

The percentage of PVN magnocellular oxytocin-immunoreactive cells expressing c-Fos was significantly higher in the SCM gavage group in comparison with the balloon gavage group (balloon gavage, 8 ± 2%; SCM gavage, 26 ± 6%; $P = 0.03$) [Fig. 2(c) and 2(e)], and expression in the sham group was not significantly different from either SCM or balloon gavage group (sham gavage, 16 ± 5%; $P = 0.7$ and $P = 0.5$ for balloon and SCM respectively) [Fig. 2(c)]. There was no significant difference in c-Fos expression in PVN parvocellular oxytocin neurons between the sham, balloon, and SCM gavage groups (sham gavage, 35 ± 6%; balloon gavage, 32 ± 5%; SCM gavage, 35 ± 5%; $P = 0.9$) [Fig. 2(d)].

In the NTS, there were no significant differences in c-Fos expression between the groups (sham gavage, 238 ± 22 nuclei/section; balloon gavage, 286 ± 33 nuclei/section; SCM gavage, 247 ± 13 nuclei/section; $P = 0.4$) or in the percentage of NTS TH-immunoreactive cells expressing c-Fos (sham gavage, 36 ± 6%; balloon gavage, 43 ± 4%; SCM gavage, 41 ± 4%; $P = 0.5$).

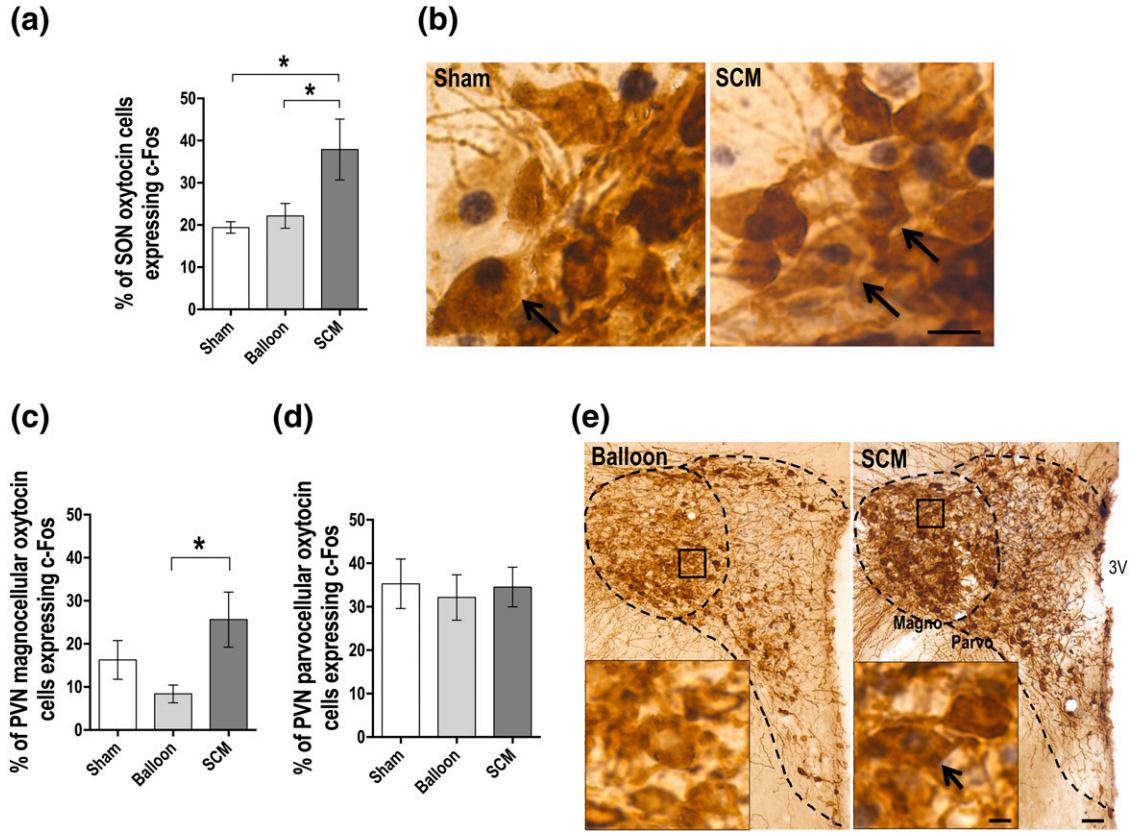

Figure 2. c-Fos–like immunohistochemistry in oxytocin neurons within the SON and PVN in response to sham, balloon, or SCM gavage. Data shown as means ± standard error of the mean. *$P < 0.05$. (a) Percentage of SON oxytocin-immunoreactive cells expressing c-Fos–positive nuclei per brain section for sham, balloon, and SCM gavage groups [$P = 0.01$, one-way analysis of variance (ANOVA) with Bonferroni]. (b) Representative images of c-Fos expression within SON oxytocin-immunoreactive cells for sham and SCM gavage groups. Bregma −0.84 mm (26). Scale bar represents 10 μm. (c) Percentage of PVN magnocellular oxytocin-immunoreactive cells expressing c-Fos–positive nuclei per brain section for sham, balloon, and SCM gavage groups ($P = 0.04$, one-way ANOVA with Bonferroni). (d) Percentage of PVN parvocellular oxytocin-immunoreactive cells expressing c-Fos–positive nuclei per brain section for sham, balloon, and SCM gavage groups ($P = 0.9$, one-way ANOVA). (e) Representative images of c-Fos expression within PVN oxytocin-immunoreactive cells for balloon and SCM gavage groups. Bregma −1.72 mm (26). Main scale bar represents 50 μm; inset scale bar represents 10 μm. 3V, 3rd ventricle; Magno, magnocellular PVN; Parvo, parvocellular PVN.

In the ARC and VMH, there were no significant differences in c-Fos expression between groups (ARC: sham gavage, 49 ± 5 nuclei/section; balloon gavage, 36 ± 6 nuclei/section; SCM gavage, 57 ± 5 nuclei/section; $P = 0.051$; VMH: sham gavage, 26 ± 6 nuclei/section; balloon gavage, 16 ± 4 nuclei/section; SCM gavage, 35 ± 6 nuclei/section; $P = 0.06$).

Experiment 3: Blood sampling during SCM or cream gavage in anesthetized rats

We measured blood glucose, plasma insulin, plasma osmolarity, and plasma sodium at −20, 0, 20, and 60 minutes with respect to start of sham, SCM, or single cream gavage. In the SCM gavage group, blood glucose increased 20 minutes after the start of gavage and was significantly increased after 60 minutes ($P = 0.01$ compared with $t = -20$ minutes) [Fig. 3(a)]. Blood glucose levels did not change with time in the sham or cream gavage groups ($P = 0.91$ and $P = 0.11$, respectively) [Fig. 3(a)]. In the SCM gavage group, plasma insulin increased 60 minutes after the start of gavage; however, this increase was not significant ($P = 0.24$) [Fig. 3(b)]. In the cream gavage group, plasma insulin was significantly increased 20 and 60 minutes after the start of gavage ($P = 0.04$ and $P = 0.01$, respectively, compared with $t = -20$ minutes) [Fig. 3(b)]. Plasma insulin did not change with time in the sham gavage group ($P = 0.91$) [Fig. 3(b)]. There were no significant differences in plasma osmolarity over time in the sham, SCM, or cream gavage groups ($P = 0.61$, $P = 0.51$, and $P = 0.23$, respectively) [Fig. 3(c)]. There were also no significant differences in plasma sodium over time in the sham, SCM, or cream gavage groups ($P = 0.91$, $P = 0.99$, and $P = 0.65$, respectively) [Fig. 3(d)].

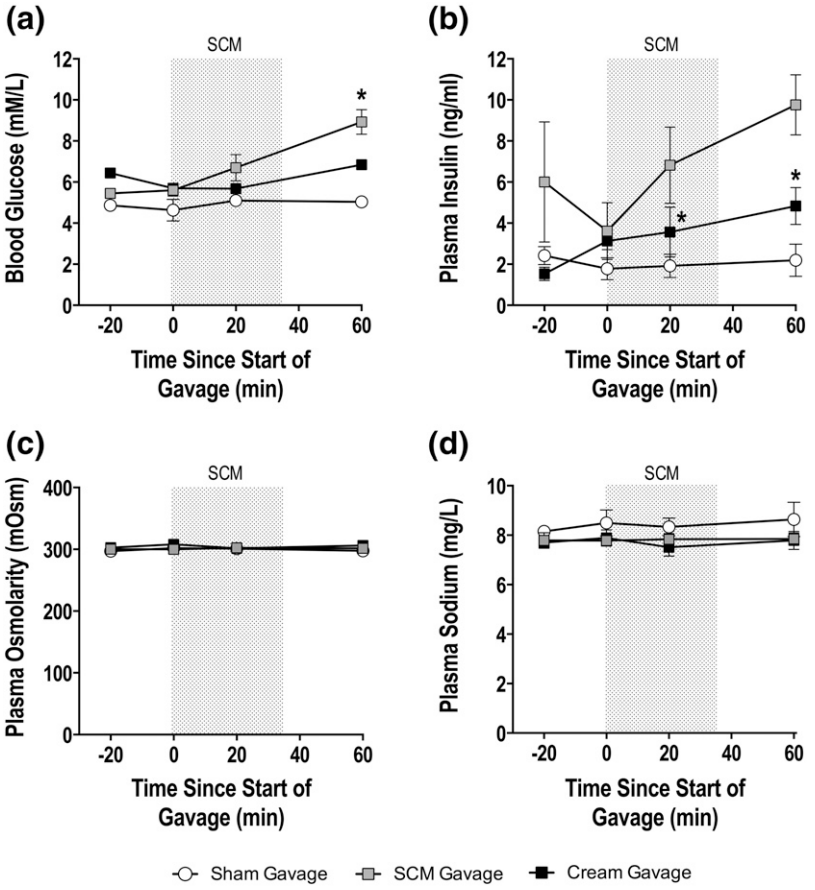

Figure 3. Blood glucose, plasma insulin, plasma osmolarity, and plasma sodium at time points −20, 0, 20, and 60 minutes with respect to the start of sham, SCM, or single cream gavage. Data shown as means ± standard error of the mean. (a) Time course of blood glucose (mM/L) for sham, SCM, and cream gavage groups. $*P < 0.05$ [Friedman test with Dunn multiple comparisons for the SCM ($P = 0.0006$) gavage group over time; Friedman test for sham ($P = 0.91$) or cream ($P = 0.11$) gavage groups over time]. (b) Time course for plasma insulin (ng/mL) for sham, SCM, and cream gavage groups. $*P < 0.05$ [Friedman test with Dunn multiple comparisons for the cream gavage group over time ($P = 0.0087$); Friedman test for SCM ($P = 0.24$) or sham ($P = 0.91$) gavage groups over time]. (c) Time course of plasma osmolarity (mOsm) for sham, SCM, or cream gavage groups [Friedman test for the sham ($P = 0.61$), SCM ($P = 0.51$), or cream ($P = 0.23$) gavage groups over time]. (d) Time course of plasma sodium (mg/L) for sham, SCM, or cream gavage groups [Friedman test for the sham ($P = 0.91$), SCM ($P = 0.99$), or cream ($P = 0.65$) gavage groups over time].

Experiment 4: *In vivo* electrophysiology during SCM or cream gavage in anesthetized rats

Rats were fasted overnight and anesthetized with urethane. For SCM gavage, we recorded data from nine SON oxytocin neurons and nine SON vasopressin neurons (of which five fired phasically and four fired continuously). Every nonphasic cell was tested with an intravenous injection of CCK after the gavage. Cells that were activated by CCK were classed as oxytocin cells [Fig. 4(a)], whereas cells that were inhibited were considered vasopressin cells. During single cream gavage, we recorded from six SON oxytocin neurons.

After 10 minutes of stable, basal recording of the mean firing rate in a single cell, we started gavage of 5 mL SCM or cream directly into the stomach and recorded the effect of firing rate throughout the gavage, which lasted about 35 minutes. In most cells, recordings were maintained up to 20 minutes after the gavage had finished.

At the beginning of SCM gavage, the mean firing rate of the nine oxytocin cells was 4.9 ± 1.2 spikes/s. Seven of these cells showed an increase in mean firing rate within 10 minutes of the start of gavage. Between 10 and 20 minutes after the start of gavage, the overall mean rate was significantly higher than the basal rate [Fig. 4(b)].

The mean firing rate increased linearly with duration of gavage, with a slope of 0.042 spikes/s/min (R^2 = 0.9) [Fig. 4(c)]. This increase lasted until the end of gavage, reaching a plateau between 40 and 60 minutes after the start of gavage. The mean change averaged over the 50-minute period between 10 and 60 minutes after gavage started was 1.2 ± 0.3 spikes/s (P = 0.004). In response to CCK, the nine cells increased their firing rate by 1.3 ± 0.24 spikes/s in the first 5 minutes after injection [Fig. 4(d)]; the magnitude of the response to CCK was not significantly correlated with the response to gavage (R^2 = 0.02).

In vasopressin cells, we combined the recordings from phasic-firing [Fig. 5(a)] and continuous-firing cells and analyzed the mean difference in firing rate in 10-minute bins in response to SCM gavage. Seven of the nine cells increased their firing rate within 10 minutes of the start of the gavage. Between 10 and 20 minutes after the start of gavage, the overall mean rate was significantly higher than the basal rate. The mean change averaged over the 50-minute period between 10 and 60 minutes after gavage started was +1.4 ± 0.5 spikes/s (from a mean basal firing rate of 5.4 ± 0.5 spikes/s; P = 0.008) [Fig. 5(b)].

During cream gavage, four of the six oxytocin cells were responsive. The change in firing rate in response to gavage exceeded 0.5 Hz. In these cells, there was a decrease in the mean firing rate within 5 minutes of cream gavage, and the overall mean rate was significantly lower than the basal rate between 5 and 30 minutes after the start of gavage, reaching a maximum decrease at 30 minutes before starting to recover its original level ($P < 0.001$) [Fig. 6(a)]. Two of the six cells were considered unresponsive to single cream gavage (mean change in firing rate <0.5 Hz). Overall, before gavage, the mean basal firing rate in the six oxytocin cells was 2.4 ± 0.52 spikes/s, and the mean change averaged over the 25-minute period between 5 and 30 minutes after gavage started was –0.57 ± 0.31 spikes/s [Fig. 6(b)]. This decrease in firing rate in response to cream in the averaged six cells was not significant (P = 0.156).

Discussion

We studied the involvement of oxytocin neurons in the response to food consumption or gavage using c-Fos immunoreactivity and *in vivo* electrophysiology. Satiated rats that voluntarily consumed SCM showed increases in c-Fos expression in magnocellular SON and PVN neurons. To avoid confounding from oro-sensory signaling (12), we used oral gavage to instill SCM directly into the stomach and found the same pattern of c-Fos expression in the SON and PVN. We confirmed previous

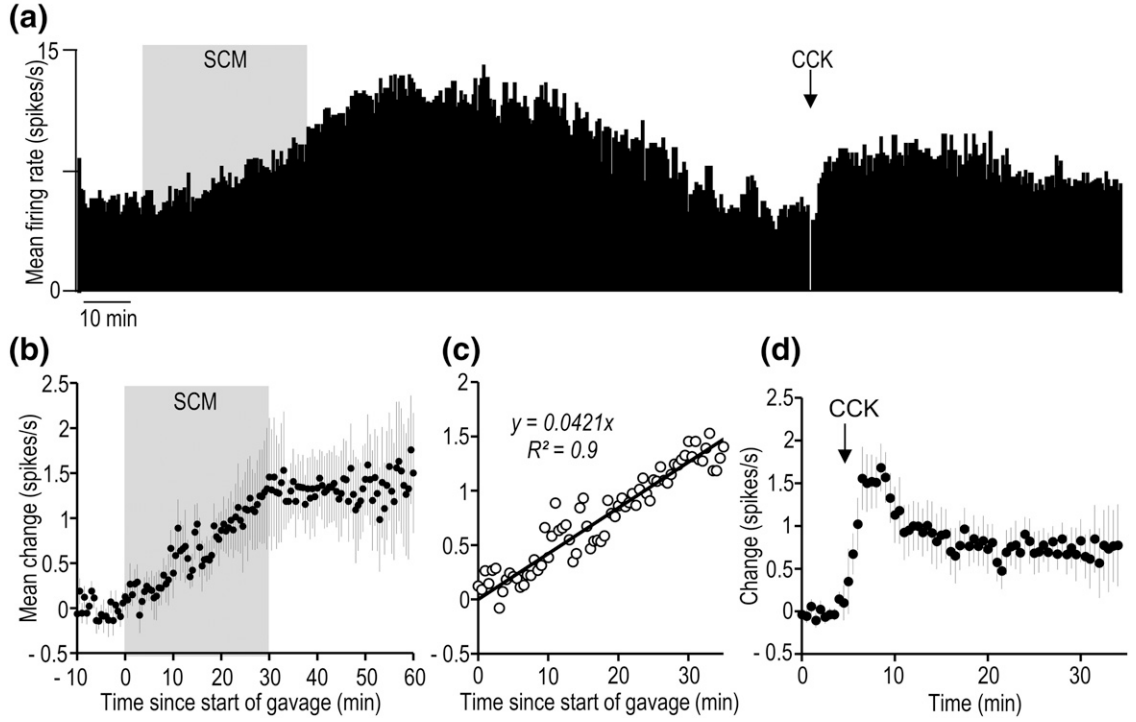

Figure 4. Effects of SCM gavage on the electrical activity of oxytocin neurons in the SON of rats *in vivo*. (a) Typical example of the increase in the mean firing rate in an oxytocin cell in response to SCM gavage and excitation in response to subsequent intravenous injection of CCK. (b) Mean changes in the firing rate (in 30-second bins) in oxytocin cells in response to gavage of SCM [n = 9; ± standard error of the mean (SEM)]. (c) The linear regression line fitted to the mean changes in firing rate plotted in b. (d) Mean change in firing rate in 30-second bins in the oxytocin cells in response to CCK (n = 9; ± SEM).

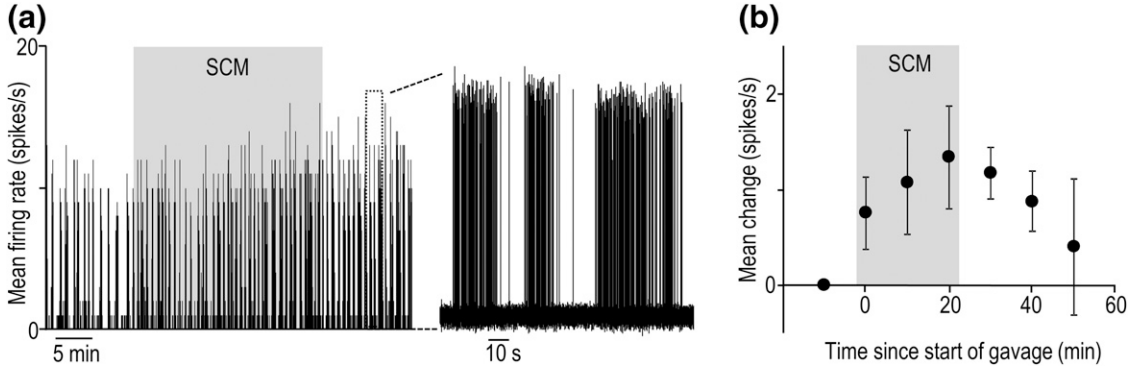

Figure 5. Effects of SCM gavage on the electrical activity of vasopressin neurons in the SON of rats *in vivo*. (a) Example of the response of a phasically firing vasopressin neuron in response to SCM gavage (firing rate in 10-second bins) with extract of raw voltage signal showing recorded action potentials. (b) Mean change in firing rate in vasopressin cells in 10-minute bins in response to SCM gavage (n = 9; ± standard error of the mean).

observations of c-Fos protein expression in the PVN after voluntary consumption of food (29) and extended observations of c-Fos mRNA expression in the SON after voluntary consumption or conscious gavage of SCM (12). We used double-label immunohistochemistry for c-Fos and oxytocin and found increased c-Fos expression in SON and PVN magnocellular oxytocin neurons after voluntary consumption or gavage of SCM.

Gastric distention has been shown to induce SON c-Fos immunoreactivity and to increase the electrical activity of SON oxytocin neurons (14, 27). With this in mind, we controlled for distention by inflating a balloon at the same rate and volume as in food gavage. We found no differences in c-Fos immunoreactivity in SON oxytocin neurons between the sham and balloon gavage groups. We did not record the electrical activity of SON oxytocin neurons in response to gastric distention, but the initial increase in firing rate with SCM gavage is unlikely due to gastric distention as the electrical response occurs very soon after gavage onset, before appreciable distention can occur.

In the rat, oxytocin neurons are osmosensitive (30) and receive input from osmosensitive brain regions (31), and oxytocin is secreted peripherally in response to osmotic stimuli and promotes sodium excretion (32, 33). We did not measure plasma oxytocin in this study, but neither plasma osmolarity nor plasma sodium was altered by SCM gavage, so it is unlikely that these served as stimuli for the observed increase in firing rate.

The expression of c-Fos in a given cell does not necessarily reflect a change in electrical activity (20). To study electrical responses directly, we used *in vivo* electrophysiology and identified SON oxytocin neurons pharmacologically. Consistent with the c-Fos mapping studies, the firing rate of SON oxytocin neurons increased during gavage of SCM, a high-sugar food. However, gavage of isocaloric cream was not associated with an increase in firing rate, showing that high-sugar, but not high-fat, food in the stomach increases the electrical activity of oxytocin neurons.

We observed a strong association between the time since the start of SCM gavage and the mean firing rate of oxytocin neurons. These neurons responded very rapidly to the onset of SCM gavage, their response was linear and proportional to the amount of SCM gavaged, and the

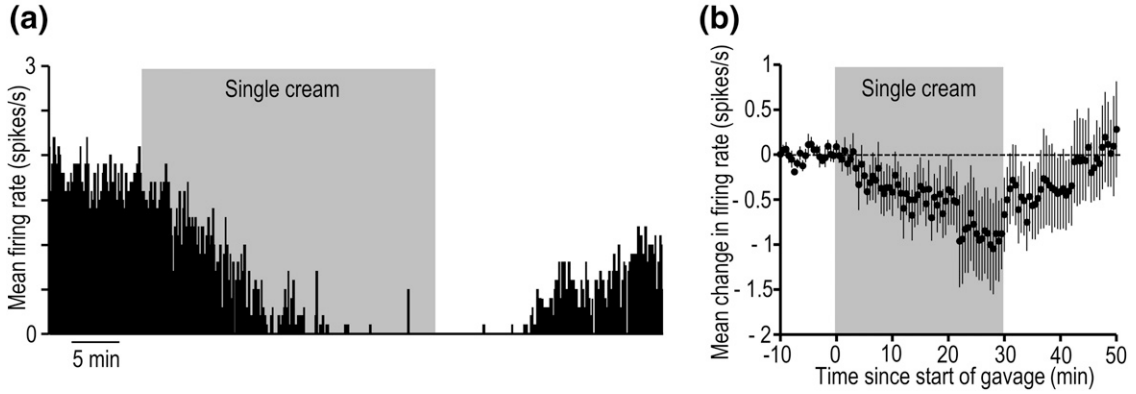

Figure 6. Effects of single cream gavage on the electrical activity of oxytocin neurons in the SON of rats *in vivo*. (a) Typical example of the response of an oxytocin cell to single cream gavage. (b) Mean change in firing rate (in 30-second bins) in oxytocin cells in response to single cream gavage (n = 6; ± standard error of the mean).

increased firing rate persisted after the offset of gavage. This *in vivo* electrophysiological response is similar to that seen in rat oxytocin and vasopressin SON neurons during infusion of hypertonic saline (34) and provides further evidence for a role for these neurons in accurately encoding variations in physiological stimuli.

There is emerging evidence for SON oxytocin neurons as "metabolic sensors." The SON expresses insulin receptors and glucokinase, an enzyme considered to play a critical role in glucose-sensitive cells of the brain, pancreas, and liver. In an *in vitro* model, glucose and insulin stimulated oxytocin release from explants containing the SON (35, 36). However, in *in vitro* slice preparations, SON neurons do not increase their firing rate in response to increased glucose in the perifusion medium (30), and intravenous infusion of glucose results in an increase in c-Fos expression in the rat PVN but not SON (37). In our study, we did not measure cerebrospinal fluid glucose levels; however, after SCM gavage, plasma glucose levels began to increase soon after the onset of gavage and were significantly increased 60 minutes after the start of gavage. Firing rates in oxytocin cells showed a similar pattern, reaching a maximum around 30 minutes after the onset of gavage. In addition, we observed no change in blood glucose after cream gavage and no increase in firing rates in oxytocin cells.

We also measured plasma insulin levels during gavage. There was a large variation in values at −20 minutes in the SCM group, but plasma insulin levels appear to increase during SCM gavage. After cream gavage, plasma insulin levels were also increased but not to the same extent. These data support the idea that increases in glucose or insulin may be an important stimulus for the oxytocin system. It would be of interest to measure cerebrospinal fluid glucose and insulin levels during food gavage and to determine whether centrally or peripherally administered glucose or insulin given at physiologically relevant doses affects the firing rate of SON neurons.

Magnocellular oxytocin neurons from the SON and PVN can secrete oxytocin into the circulation from projections to the posterior pituitary and can independently secrete centrally via dendritic release (2, 20). If specific activation of the magnocellular system by SCM gavage is sufficient to stimulate oxytocin release, both peripheral and central oxytocin secretion may occur. We did not measure plasma oxytocin, but there is a small body of evidence suggesting that oxytocin is released peripherally in response to food consumption: plasma oxytocin increases after the onset of feeding in food restricted rats and in response to intragastric infusion of milk in 10-day-old rat pups (38, 39). Given the apparent role for magnocellular cells and an apparent lack of activation of parvocellular cells in our model, it would be of interest to determine whether oxytocin is released centrally and/or peripherally in response to food in the stomach and to determine which sites of action, both in the brain and in peripheral tissues, are involved in this response. One potential brain target is the VMH. The VMH contains oxytocin binding sites (40), and targeted administration of oxytocin to the VMH results in increased energy expenditure and reduced feeding (1). We observed a nonsignificant increase in c-Fos expression in the VMH after SCM gavage, but it is not clear whether this represents a direct downstream effect of central oxytocin release after SCM gavage or is related more broadly to satiety signaling (41).

Putative SON vasopressin neurons showed a small, nonsignificant increase in c-Fos expression with voluntary SCM consumption and SCM gavage. We also showed a transient increase in the firing rate of pharmacologically identified vasopressin neurons during SCM gavage. This response was qualitatively different from that seen in oxytocin cells. Firing rates in vasopressin cells increased during SCM gavage, but this increase was not sustained at the offset of gavage. An increase in c-Fos immunoreactivity in SON vasopressin neurons in response to refeeding after food restriction was previously noted (11). However, this is likely due to an osmotic stimulus (42). We have shown that plasma osmolarity and plasma sodium concentrations do not change with SCM gavage. Therefore, an osmotic effect is unlikely to underlie the response of vasopressin or oxytocin neurons to SCM ingestion in our experiments. There is little evidence for a role of vasopressin neurons in appetite control (43, 44). The mechanisms underlying a potential role of vasopressin in appetite control remains unknown; unlike oxytocin neurons, SON vasopressin neurons do not appear to be innervated by NTS projections and are inhibited by the satiety signal CCK (28).

There are multiple mechanisms by which gut-initiated signaling could influence the activity of oxytocin neurons. It is possible that the increase in oxytocin neuron activity in response to SCM gavage is mediated by CCK, a peptide signal released in response to nutrients entering the duodenum of the small intestine. CCK acts via vagal afferents to activate noradrenergic projections from the NTS to magnocellular oxytocin neurons, resulting in oxytocin release from the posterior pituitary (45–47). We quantified c-Fos immunoreactivity in the NTS and in particular in neurons immunoreactive for TH. In rats that consumed SCM voluntarily, we saw an increase in c-Fos expression within TH-immunoreactive cells in the NTS. Therefore, it is possible that, after the consumption of SCM, CCK secreted from the gut activates vagal afferents to activate noradrenergic neurons in the NTS. Activation

of noradrenergic NTS projections to the SON and PVN may subsequently influence the activity of oxytocin neurons.

Glucagonlike peptide 1 (GLP-1) is also released from the gut in response to nutrients and is thought to mediate a satiety effect through vagal neurons and the NTS (48). The effects of peripheral GLP-1 on the activity of oxytocin neurons have not been well characterized; however, peripheral administration of GLP-1 results in c-Fos expression in the SON (49), whereas central injection of GLP-1 induces c-Fos expression in both PVN and SON magnocellular neurons (50). There is evidence for complex effects of GLP-1 on peripheral oxytocin release that depend on the model system and the dose and route of administration (51, 52). There is also evidence for central sources of GLP-1 (53), and behavioral studies using central administration in rats have resulted in opposing effects of blockade of GLP-1 receptors on the anorexigenic effects of oxytocin administration (51, 54). However, oxytocin receptor blockade does not prevent GLP-1–induced reductions in food intake (51). Given our observations of differing electrophysiological responses using isocaloric foods with different macronutrient profiles, it would be of interest to determine the signaling mechanisms that appear to detect different macronutrients and convey information about the macronutrient profile from the gut to the brain.

The activity of oxytocin neurons could also be influenced by centrally released appetite-associated signals. ARC pro-opiomelanocortin neurons project to both the SON and PVN (55), which express the melanocortin MC4 receptor (56). Many studies demonstrate that melanocortin signaling, particularly in the PVN, plays a fundamental role in appetite control (57). Despite this, a recent study has shown that re-expression of the MC4 receptor, specifically in PVN oxytocin neurons in MC4 knock-out mice, does not reverse the obesity phenotype seen in these animals (58). However, this study does not address a potential role for SON oxytocin neurons. We have previously shown that central administration of the endogenous MC4 receptor agonist α-MSH increases c-Fos expression in SON oxytocin neurons and induces central release of oxytocin from their dendrites (20). In that study we also showed that α-MSH inhibits the electrical activity of SON oxytocin neurons and peripheral oxytocin secretion from the posterior pituitary. Therefore, the importance of peripheral and central oxytocin secretion in response to food consumption remains unclear.

In our gavage experiments, it is possible that taste-independent, postingestive reward signaling may influence the activity of oxytocin neurons. Neither the ventral tegmental area (59) nor the nucleus accumbens (60) of the mesolimbic reward system directly project to the SON or PVN in the rat. However, other appetite-associated brain regions implicated in communicating with the reward system, for example the NTS, project to the SON and PVN. Therefore, reward signaling after food ingestion may indirectly activate oxytocin neurons.

We also quantified c-Fos expression in a number of other appetite-related brain regions. It has been shown that c-Fos expression increases in the ARC, VMH, and NTS after refeeding in fasted rats (11). We did not observe this in our model. However, it would be of interest to determine whether specific cell types within these regions respond to SCM gavage, particularly given the potential role of the VMH as a target for oxytocin signaling.

In summary, we provide evidence for differential activation of SON oxytocin neurons by isocaloric foods in the stomach. We observed an increase in electrical activity in these cells after gavage of high-sugar, but not high-fat, food. Activation is independent of oro-sensory signaling, gastric distention, and plasma osmolarity or plasma sodium levels. SON oxytocin cells show a remarkably rapid, linear, and proportional excitatory response during SCM gavage, and this response is stable and sustained after the end of gavage. This indicates these cells may have a dynamic role in appetite signaling.

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